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New Inhibitors of Platelet Aggregation. 5′-Phosphate, 5′-Phosphorothioate, and 5′-*O*-Sulfamoyl Derivatives of 2-Substituted Adenosine Analogues

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Analogues of AMP incorporating modifications of the adenine moiety and the phosphate function were synthesized as potential inhibitors of platelet aggregation. 2-Methoxy-, 2-ethoxy-, 2-methylthio-, 2-ethylthio-, 2-methylamino-, 2-ethylamino-, 2-trifluoromethyl-, 2-chloro-N^6-methyl-, and 2-methylthio-N^6-methyladenosines were converted, via 2′,3′-*O*-isopropylidene derivatives, to the 5′-monophosphates using 2-cyanoethyl phosphate and DCC. The isopropylidene derivatives of adenosine, 2-chloroadenosine, and 2-methylthioadenosine were also used to synthesize the respective 5′-*O*-sulfamoyladenosines by reaction with NaH and sulfamoyl chloride and subsequent deblocking. In addition, 2-chloroadenosine 5′-phosphorothioate, 2-methylthioadenosine 5′-phosphorothioate, and 2-ethylthioadenosine 5′-phosphorothioate were prepared from the unprotected nucleosides by treatment with $PSCl_3$ in triethyl phosphate. With the exception of the 5′-*O*-sulfamates of adenosine and 2-chloroadenosine, all the compounds tested inhibited the ADP-induced aggregation of sheep platelets. The 5′-phosphates and phosphorothioates of 2-methylthio- and 2-ethylthioadenosine were 2–13 times more potent than adenosine; the remaining 2- and N^6-substituted phosphates and phosphorothioates were less potent than adenosine. 5′-*O*-Sulfamoyladenosine and 2-chloro-5′-*O*-sulfamoyladenosine potentiated ADP-mediated platelet aggregation but the three 5′-*O*-sulfamates inhibited serotonin-induced platelet aggregation. In contrast, all the 5′-phosphate and 5′-phosphorothioate analogues tested had negligible activity as inhibitors of serotonin-induced sheep platelet aggregation.

The aggregation of blood platelets in response to vascular insult plays a central role in the formation of arterial thrombi. Collagen exposed by injury to blood vessels, and thrombin and ADP (adenosine 5′-diphosphate) produced as a result of such injury, are the primary agents which cause platelets to clump together in vivo.[1-3] In vitro, platelet aggregation can be induced by a variety of stimuli including collagen, thrombin, serotonin, ADP,[1] and 2-substituted ADP analogues.[4] Drugs that inhibit platelet aggregation have potential for use both as antithrombotic

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Table I. 2-Substituted Isopropylideneadenosines

2',3'-O-Isopropylideneadenosine	Mp, °C	R_f in solvent I	R_f in solvent II	Formula (analyses)
2-Methoxy- (**2c**)	169-170	0.71 (0.31)[b]	0.71	$C_{14}H_{19}N_5O_5$ (C, H, N)
2-Ethoxy- (**2d**)	95-99	0.80 (0.52)	0.74	$C_{15}H_{21}N_5O_5 \cdot 0.75H_2O$ (C, H, N)
2-Methylthio- (**2e**)	178-179	0.75 (0.35)	0.71	$C_{14}H_{19}N_5O_4S$ (C, H, N)
2-Ethylthio- (**2f**)	134-138	0.83 (0.63)	0.79	$C_{15}H_{21}N_5O_4S \cdot 0.25H_2O$ (C, H, N)
2-Methylamino- (**2g**)	182.5-183.5	0.70 (0.32)	0.70	$C_{14}H_{20}N_6O_4$ (C, H; N[c])
2-Ethylamino- (**2h**)	*a*	0.73 (0.51)	0.76	$C_{15}H_{22}N_6O_4 \cdot 0.75H_2O$ (C, H, N)
2-Trifluoromethyl- (**2i**)	105-107	0.82 (0.62)	0.86	$C_{14}H_{16}F_3N_5O_4 \cdot H_2O$ (C, H, N)
2-Chloro-N^6-methyl- (**2j**)	163-164	0.85 (0.63)	0.79	$C_{14}H_{18}ClN_5O_4$ (C, H, N)
2-Methylthio-N^6-methyl- (**2k**)	173.5-174.5	0.85 (0.51)	0.83	$C_{15}H_{21}N_5O_4S$ (C, H, N)

[a] Could not be crystallized. Analytically pure material was obtained as a freeze-dried solid after preparative paper chromatography (solvent, aqueous NH_4OH, pH 10). [b] R_f values of the parent nucleosides **1c-k** are given in parentheses. [c] N: calcd, 25.0; found, 25.5.

Scheme I

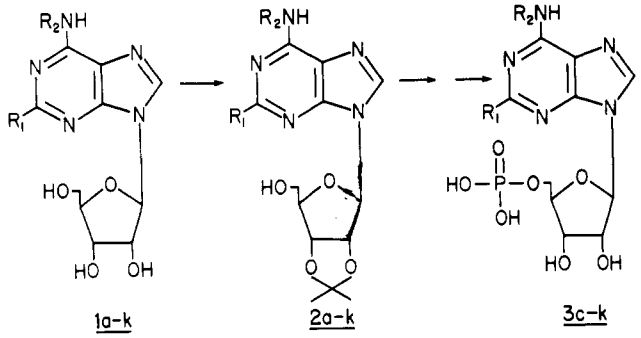
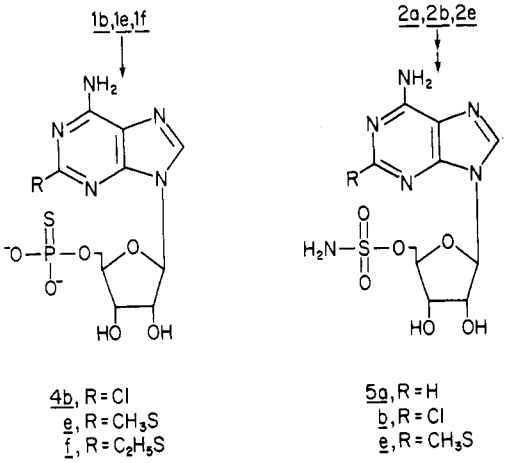


Chart I



agents and as tools with which to study the mechanism and sequence of events of the platelet aggregation process induced by the different aggregating agents. We reported that the AMP (adenosine 5'-monophosphate) analogue, 2-methylthioadenosine 5'-monophosphate, was a potent inhibitor of ADP-induced platelet aggregation and that semi-in vivo studies indicated the possible usefulness of the analogue as an antithrombotic agent.[5] Another analogue, 2-chloroadenosine 5'-monophosphate, and unsubstituted AMP were much less active as inhibitors of platelet aggregation.[6] Our investigations of AMP analogues as inhibitors of platelet aggregation have been extended to include a series of new 2- and N^6-substituted AMP derivatives. Also examined were AMP analogues in which the phosphate moiety of AMP is replaced by the phosphate isosteres, phosphorothioate and sulfamate, and several derivatives which incorporate both substitution at the 2 position of adenine and isosteric modification of the phosphate function. We report here the syntheses of these analogues and a summary of their effects on ADP- and serotonin-induced sheep platelet aggregation.

Chemistry. Nucleosides **1c-k** were converted to their 2',3'-*O*-isopropylidene derivatives **2c-k** in 85-100% yields by acid-catalyzed reaction with acetone, following the method of Hampton[7] (see Scheme I and Table I). Thus protected, they were phosphorylated at the 5'-hydroxyl using 2-cyanoethyl phosphate and DCC, after Tener.[8] Treatment with LiOH and then with H_3PO_4 removed blocking groups from the resulting isopropylidene nucleoside 5'-cyanoethyl phosphates. The nucleotide products **3c-k** were freed from excess phosphate, purified by passage through cation- and anion-exchange columns, and crystallized as the free acids in yields of 47-95%.

Phosphorylation of the unprotected nucleosides **1b,e,f** with $PSCl_3$ in triethyl phosphate, according to the procedure of Murray and Atkinson,[9] gave the corresponding phosphorothioates **4b,e,f** which were isolated as their ammonium salts in 26-42% yields following DEAE cellulose chromatography (see Chart I and Table II).

5'-*O*-Sulfamoyladenosine has been prepared by Shuman et al.[10] from 2',3'-*O*-ethoxymethylideneadenosine. However, the ortho ester function was found to be inconveniently labile when attempts were made to use it as a protecting group for the *cis*-hydroxyls of the 2-substituted adenosines **1b** and **1e**,[11] perhaps due to the significantly lower basicity of the purine moieties of these nucleosides. Consequently, the 5'-*O*-sulfamoyl derivatives **5a,b,e** were synthesized by allowing the isopropylidene nucleosides **2a,b,e** to react with NaH and sulfamoyl chloride. After removal of the blocking group, the sulfamates were isolated in 39-53% yields (see Chart I and Table III).

Platelet Aggregation. Sheep blood was collected by jugular venipuncture into sufficient 7.6% sodium citrate to give a final concentration of 0.38% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of the citrated blood for 24 min at 140g. Platelet-free plasma (PFP) was obtained by centrifuging the residual blood or PRP at 1000g for 10 min. Platelet counts were performed by the Brecker-Cronkite method[12] and PRP preparations were diluted if necessary with PFP to give 300 000-400 000 platelets/mm^3. Platelet aggregation was measured at 37 °C by the photometric method of Born[13] in 3.0- or 0.5-mL PRP samples, monitoring either the increase in light transmission or the decrease in optical density associated with platelet aggregation. Aggregating agents and inhibitors were dissolved in normal saline buffered to pH 7.4 with sodium barbitone. Additions to

Table II. 5′-Substituted Adenosine Analogues

Compd	R_f in solvent II	R_f in solvent III	M_{AMP}[a]	λ_{max} (0.1 N HCl), nm ($\epsilon \times 10^{-3}$)	Formula (analyses)
A. 5′-Dihydrogen Monophosphates					
2-Methoxyadenosine 5′-phosphate (**3c**)	0.20	0.64	0.94	251, 273 (6.65, 11.3)	$C_{11}H_{16}N_5O_8P \cdot H_2O$ (C, H, N, P)
2-Ethoxyadenosine 5′-phosphate (**3d**)	0.32	0.74	0.94	251, 274 (6.95, 11.5)	$C_{12}H_{18}N_5O_8P \cdot HCOOH$ (C, H, N, P)
2-Methylthioadenosine 5′-phosphate (**3e**)	0.20	0.64	0.85	268 (16.7)	$C_{11}H_{16}N_5O_7PS \cdot H_2O$ (C, H, N, P)
2-Ethylthioadenosine 5′-phosphate (**3f**)	0.32	0.73	0.87	270 (16.9)	$C_{12}H_{18}N_5O_7PS \cdot 2HCOOH$ (H, N, P; C[b])
2-Methylaminoadenosine 5′-phosphate (**3g**)	0.17	0.74	0.49	255, 298 (12.0, 8.57)	$C_{11}H_{17}N_6O_7P \cdot 2.25H_2O$ (C, H, N; P[c])
2-Ethylaminoadenosine 5′-phosphate (**3h**)	0.22	0.83	0.70	254.5, 298 (13.9, 8.75)	$C_{12}H_{19}N_6O_7P \cdot 0.5H_2O$ (C, H, N, P)
2-Trifluoromethyladenosine 5′-phosphate (**3i**)	0.50	0.71	0.98	260 (12.3)	$C_{11}H_{13}F_3N_5O_7P \cdot C_3H_8O$ (C, H, N, P)
2-Chloro-N^6-methyladenosine 5′-phosphate (**3j**)	0.27	0.61	0.97	272 (15.2)	$C_{11}H_{15}ClN_5O_7P \cdot 0.25H_2O$ (C, H, N, P)
2-Methylthio-N^6-methyladenosine 5′-phosphate (**3k**)	0.33	0.66	0.92	271 (16.1)	$C_{12}H_{18}N_5O_7PS \cdot H_2O$ (C, H; N[d])
B. 5′-Phosphorothioates					
2-Chloroadenosine 5′-phosphorothioate (**4b**)	0.24	0.44	1.16	264 (12.6)	$C_{10}H_{13}ClN_5O_6PS \cdot NH_3 \cdot 2H_2O$ (C, H, N, P)
2-Methylthioadenosine 5′-phosphorothioate (**4e**)	0.22	0.53	0.96	268 (14.2)	$C_{11}H_{16}N_5O_6PS_2 \cdot NH_3 \cdot 1.75H_2O$ (C, H, N, P)
2-Ethylthioadenosine 5′-phosphorothioate (**4f**)	0.33	0.75	1.06	270 (16.7)	$C_{12}H_{18}N_5O_6PS_2 \cdot NH_3 \cdot 3H_2O$ (C, N, P; H[e])
C. 5′-*O*-Sulfamoyl Derivatives					
5′-*O*-Sulfamoyladenosine (**5a**)	0.21	0.50			
2-Chloro-5′-*O*-sulfamoyladenosine (**5b**)	0.37	0.63		263.5 (14.3)	$C_{10}H_{13}ClN_6O_6S \cdot 0.25H_2O$ (C, H, N)
2-Methylthio-5′-*O*-sulfamoyladenosine (**5e**)	0.38	0.64		273 (19.2)	$C_{11}H_{16}N_6O_6S_2 \cdot H_2O$ (C, H, N)

[a] Electrophoretic mobility relative to AMP. [b] C: calcd, 33.67; found, 33.17. [c] P: calcd, 7.43; found, 6.96. [d] N: calcd, 16.4; found, 15.7. [e] H: calcd, 5.50; found, 4.89.

Table III. 5′-*O*-Sulfamoyladenosines

Starting material	Amt, mmol	NaH, mmol	Sulfamoyl chloride, mmol	Product	Yield, %	Mp, °C
Isopropylideneadenosine (**2a**)	1.67	8	3.3	**5a**	39	170 dec[a] (softens 150–152)
2-Chloroisopropylideneadenosine (**2b**)	1.0	20	10	**5b**	40	210–212
2-Methylthioisopropylideneadenosine (**2e**)	1.5	15	3	**5e**	53	177–179 (softens 138–140)

[a] Literature values:[10] softens 153–155 °C; mp 165 °C dec.

PRP samples were made in volumes of less than 100 μL for 3.0 mL of PRP and less than 15 μL for 0.5 mL of PRP; control assays without inhibitors showed that the buffered saline medium did not modify platelet responses.

The rate of aggregation was quantitated by measurement of the initial slope of the aggregation curve. Unless otherwise noted, AMP analogues were routinely incubated in stirred PRP for 2.5 min prior to the addition of ADP to give 0.67 μM ADP or serotonin to give 1.6 μM serotonin. The inhibitory potencies of the analogues were determined from plots of the log of the inhibitor dose vs. the percentage inhibition of platelet aggregation and are expressed relative to that of adenosine. Control responses to adenosine were obtained on each batch of PRP.

Results and Discussion

With the exception of 5′-*O*-sulfamoyladenosine and its 2-chloro derivative, all the AMP analogues studied inhibited ADP-mediated sheep platelet aggregation to some extent, decreasing both the initial rate and the maximum of the aggregation curve in a concentration-dependent fashion. The analogues did not modify the initial platelet shape change phase of the aggregation response. The inhibition of platelet aggregation by equimolar concentrations of adenosine, 2-ethylthioadenosine 5′-mono-

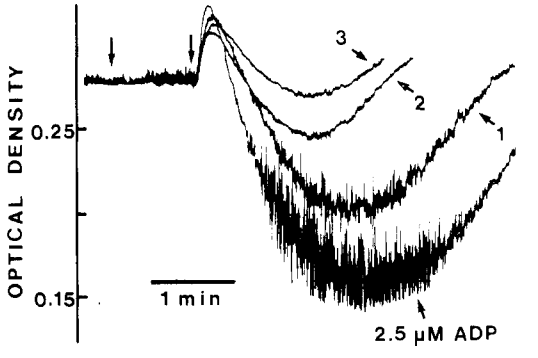


Figure 1. Inhibition of ADP-induced aggregation of sheep platelets in PRP by 3.75 μM adenosine (1), 3.75 μM 2-ethylthio-AMP (2), and 3.75 μM 2-ethylthio-AMPS (3). Inhibitors were added to PRP 1 min prior to the addition of ADP, as indicated by the vertical arrows.

Table IV. Inhibition of ADP-Induced Sheep Platelet Aggregation by Analogues of AMP[a]

Analogue	Molar potency rel to adenosine ± SE[b]
AMP[c,f]	0.12 ± 0.01
2-Methylamino-AMP (3g)	0.027 ± 0.006
2-Ethylamino-AMP (3h)	0.027 ± 0.008
2-Methoxy-AMP (3c)	0.034 ± 0.035
2-Ethoxy-AMP (3d)	0.103 ± 0.035
2-Trifluoromethyl-AMP (3i)	0.22 ± 0.05
2-Chloro-AMP[c,d]	0.12 ± 0.01
2-Methylthio-AMP (3e)	2.41 ± 1.23
2-Ethylthio-AMP (3f)	5.20 ± 0.96
2-Chloro-N^6-methyl-AMP (3j)	0.12 ± 0.03
2-Methylthio-N^6-methyl-AMP (3k)	0.46 ± 0.12
AMPS[c,e]	0.04 ± 0.01
2-Chloro-AMPS (4b)	0.44 ± 0.08
2-Methylthio-AMPS (4e)	4.0 ± 0.9
2-Ethylthio-AMPS (4f)	13.4 ± 3.4

[a] ADP concentration was 0.67 μM. [b] Means of at least four determinations. Analogues were incubated in citrated PRP for 2.5 min prior to the addition of ADP; four different doses of each analogue were used. The rate of aggregation was quantitated from the initial slope of the aggregation curve, and potency values were obtained from plots of percent inhibition vs. the log of the dose of the analogue. [c] Molar potencies increased with time when incubated for periods longer than 2.5 min prior to the addition of ADP, presumably because of the formation of the more potent parent nucleosides, adenosine and 2-chloroadenosine.[6] [d] Reference 6. [e] Reference 9. AMPS = adenosine 5′-phosphorothioate. [f] AMP = adenosine 5′-monophosphate.

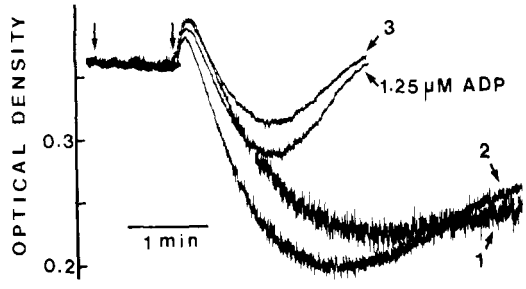


Figure 2. Effects of 100 μM 5′-*O*-sulfamoyladenosine (1), 100 μM 2-chloro-5′-*O*-sulfamoyladenosine (2), and 100 μM 2-methylthio-5′-*O*-sulfamoyladenosine (3) on ADP-induced aggregation of sheep platelets in PRP. 5′-Sulfamates were added to PRP 1 min prior to the addition of ADP, as indicated by the vertical arrows.

phosphate, and 2-ethylthioadenosine 5′-phosphorothioate is shown in Figure 1. Concentrations of the AMP and AMPS (adenosine 5′-phosphorothioate) derivatives which reduced aggregation mediated by 0.67 μM ADP gave log dose–inhibition curves parallel to that of adenosine. The potencies of these analogues as inhibitors of ADP-mediated platelet aggregation are summarized in Table IV. 5′-*O*-Sulfamoyladenosine and 2-chloro-5′-*O*-sulfamoyladenosine in concentrations up to 300 μM did not inhibit ADP-mediated platelet aggregation but, instead, enhanced the aggregation response, increasing maximum aggregation and reducing the rate of disaggregation, as shown in Figure 2. In contrast, 2-methylthio-5′-*O*-sulfamoyladenosine at similar concentrations inhibited ADP-induced platelet aggregation (Figure 2). Of the AMP analogues modified by substitution in position 2 of the purine ring, only 2-methylthio- and 2-ethylthioadenosine 5′-monophosphate were more potent than adenosine. These analogues were, *ipso facto*, much more potent than AMP. Substitution of ethoxy, chloro, or trifluoromethyl groups in position 2 of the purine ring of AMP did not greatly modify its potency,

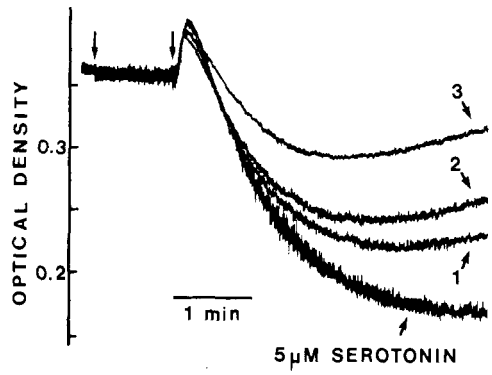


Figure 3. Inhibition of serotonin-induced aggregation of sheep platelets in PRP by 200 μM 5′-*O*-sulfamoyladenosine (1), 100 μM 2-chloro-5′-*O*-sulfamoyladenosine (2), and 200 μM 2-methylthio-5′-*O*-sulfamoyladenosine (3). Inhibitors were added to PRP 1 min prior to the addition of serotonin, as indicated by the vertical arrows.

but the substitution of the methoxy, methylamino, and ethylamino functions significantly reduced the inhibitory activity of AMP. Methylation of the exocyclic amino group in two of the 2-substituted AMP derivatives had different effects on the potencies of the analogues. 2-Chloro-N^6-methyladenosine 5′-monophosphate was equipotent with 2-chloroadenosine 5′-monophosphate, while in 2-methylthio-N^6-methyladenosine 5′-monophosphate activity was reduced to one-fifth of that of 2-methylthioadenosine 5′-monophosphate. In contrast, isosteric replacement of the phosphate function of 2-chloro-, 2-methylthio-, and 2-ethylthioadenosine 5′-monophosphate by phosphorothioate resulted in a two- to threefold enhancement of inhibitory activity in each analogue (cf. Figure 1), although unsubstituted AMPS was much less active than AMP. Moreover, the substitution of lower alkylthio groups in the 2 position of the purine ring of AMP and AMPS resulted in a consistent enhancement of inhibitory activity. Whether similar enhancement would be observed in higher alkylthio congeners is not known. 2-*n*-Amylthioadenosine 5′-monophosphate was reported to be more active than adenosine in inhibiting the ADP-induced aggregation in diluted rabbit PRP, but a potency value was not given.[14]

None of the AMP or AMPS analogues significantly inhibited serotonin-induced platelet aggregation. Conversely, the 5′-*O*-sulfamates of adenosine, 2-chloroadenosine, and 2-methylthioadenosine all inhibited serotonin-induced aggregation, decreasing both the rate and the maximum of the aggregation curve (Figure 3). These results suggest that serotonin-induced platelet aggregation is not mediated by ADP released from platelets as a result of serotonin uptake by platelets, as has been suggested.[15]

The more potent ADP antagonists, 2-methylthio- and 2-ethylthioadenosine 5′-monophosphate and 2-methylthio- and 2-ethylthioadenosine 5′-phosphorothioate, had no effect on heart rate when administered intravenously to anesthetized rats in doses up to 2.5 μmol/kg. Moreover, their molar hypotensive potencies in this preparation were less than 1% of that of adenosine. These observations suggest that the four nucleotides are relatively free of toxic cardiovascular effects and that they may therefore have potential as antithrombotic agents, albeit agents that would require administration via a parenteral route. Their effects on platelet aggregation induced by collagen, thrombin, and arachidonic acid will be reported elsewhere.[16,17]

Experimental Section

Material and Methods. Melting points were determined on a Kofler–Reichert apparatus and are uncorrected. UV spectra

were measured on a Perkin-Elmer 350 spectrophotometer. All evaporations were carried out under reduced pressure at <35 °C, and solids were dried in vacuo over P_2O_5 at room temperature.

Chromatograms were run on Whatman No. 1 paper by the ascending technique using the following solvent systems: (I) *n*-BuOH–H_2O (86:14), (II) *i*-PrOH–0.25 M NH_4HCO_3 (2:1), and (III) isobutyric acid–1 M NH_4OH (5:3). Paper electrophoresis was carried out on Schleicher and Schuell No. 2043 paper in 0.025 M citrate buffer, pH 4.8, with a potential drop of 200 V for 5 h, or on Whatman 3MM paper in 0.015 M citrate, pH 4.8, at 2000 V for 2 h. Microanalyses were performed by Dr. J. Fildes and her staff at the Department of Medical Chemistry, John Curtin School of Medical Research, Australian National University, Canberra, and by Dr. E. Challen of the Department of Organic Chemistry, University of New South Wales.

Nucleosides. Adenosine (**1a**) was a commercial sample from the Ajinomoto Co., Japan. **1e,f**,[18] **1i**,[19] and **1j,k**[20] were synthesized as previously described. **1c**,[21] **1d**, **1g**,[21] and **1h** were prepared by the reaction of **1b**[6] with MeONa, EtONa, $MeNH_2$, and $EtNH_2$, respectively.[22]

2′,3′-*O*-Isopropylideneadenosines (2a–k) were prepared from the corresponding nucleosides **1a–k** by reaction with anhydrous Me_2CO and 2,2-dimethoxypropane under the conditions described by Hampton.[6,7] The catalyst in each case was *p*-toluenesulfonic acid except for the conversions **1c** → **2c** and **1j** → **2j** where bis(*p*-nitrophenyl)phosphoric acid was employed. After removal of solvent from each neutralized reaction mixture, product was extracted from the residue with hot $CHCl_3$ in yields of 85–100%. The compounds were crystallized from water or aqueous ethanol; melting points and chromatographic data are given in Table I.

General Method for Synthesis of Nucleoside 5′-Monophosphates. 2′,3′-*O*-Isopropylidene nucleosides **2c–k** were allowed to react with 2-cyanoethyl phosphate and DCC in anhydrous pyridine for 24 h as previously described[6] for the synthesis of 2-chloroadenosine 5′-phosphate. After LiOH treatment of the reaction mixture and filtration, the alkaline filtrate was adjusted to pH 1.5 with H_3PO_4 and heated at 70–80 °C for 2 h. The solution was cooled and treated with LiOH to pH 9 and then evaporated in vacuo to a small volume. Precipitated Li_3PO_4 was removed by filtration and the filtrate and washings were passed through a column of Bio-Rad AG 50W-X4 (H^+, 2 × 30 cm). After a water wash, the nucleotide was displaced with 1 M NH_4OH. The eluate was concentrated to ca. 15 mL, applied to a column of Bio-Rad AG 2-X8 ($HCOO^-$, 2.5 × 10 cm), and followed by water (150 mL). The column was then eluted with 4 M HCOOH and the appropriate fractions were combined and vacuum evaporated to yield the nucleotide in its white, often crystalline, free acid form. The following compounds were synthesized; the scale of reaction and deviations from the above general procedure were as indicated.

2-Methoxyadenosine 5′-Phosphate (3c). (Scale of reaction: 1 mmol.) Free acid (350 mg, 89%) was isolated following anion-exchange chromatography as white microcrystals: mp 179 °C dec; recrystallized from H_2O, mp 183 °C dec.

2-Ethoxyadenosine 5′-Phosphate (3d). (Scale of reaction: 0.48 mmol.) Needles formed with mp 175–177 °C (75%); recrystallized from H_2O, mp 175–177 °C.

2-Methylthioadenosine 5′-Phosphate (3e). (Scale of reaction: 3 mmol.) Acidification of the concentrated eluate from the cation-exchange column to ca. pH 1 with 99% HCOOH resulted in immediate crystallization of the product as white needles (73%). Evaporation of the mother liquor to remove HCOOH followed by anion-exchange chromatography yielded further material (total 95%); recrystallized from H_2O, mp 192–195 °C dec.

2-Ethylthioadenosine 5′-Phosphate (3f). (Scale of reaction: 3 mmol.) Clusters of needles formed with mp 153.5–156 °C (77%); recrystallized from acidified (HCOOH) H_2O, mp 155–156 °C.

2-Methylaminoadenosine 5′-Phosphate (3g). (Scale of reaction: 1 mmol.) Evaporation of the anion-exchange column eluate yielded the chromatographically homogeneous product (322 mg, 77%) which could not be crystallized. The residue was lyophilized from solution in 2 M HCOOH to yield the analytically pure free acid as an amorphous solid.

2-Ethylaminoadenosine 5′-Phosphate (3h). (Scale of reaction: 1.25 mmol.) The limited solubility of this material in acid solution caused it to precipitate upon H_3PO_4 hydrolysis and during anion-exchange chromatography. After extensive washing of the AG 2 column with 4 M HCOOH, 468 mg (94%) of white crystalline solid, mp 190–192 °C dec, was isolated from the eluate; recrystallized from H_2O, plates, mp 192–194 °C dec.

2-Trifluoromethyladenosine 5′-Phosphate (3i). (Scale of reaction: 2.25 mmol.) The phosphorylation mixture in this one case was worked up as previously described[6] for the synthesis of 2-chloroadenosine 5′-phosphate. Thus, the alkaline filtrate obtained after the first LiOH treatment was applied to a column (3.2 × 25 cm) of Bio-Rad AG 50W-X4 (H^+) and washed through with water until no further UV-absorbing material could be eluted. The eluate was adjusted to pH 2.8 with aqueous $Ba(OH)_2$, heated under reflux for 1.5 h, and then concentrated to ca. 100 mL. $Ba(OH)_2$ was added to pH 7.5 and the precipitated barium phosphate was removed by centrifugation. The supernatant was treated with 2 vol of EtOH and the white barium salt which precipitated was collected by centrifugation, washed with EtOH, Me_2CO, and Et_2O, and dried, yielding 2 g. Chromatography in solvent III revealed the presence of two fluorescent components, one major and one minor, with R_f values of 0.71 and 0.62, respectively. The barium salt (300 mg) was converted to the ammonium form by passage through a column of Bio-Rad AG 50W-X4 (H^+, 1 × 20 cm) and neutralization of the effluent with NH_4OH. Evaporation to dryness yielded a glass (226 mg). This was dissolved in the minimum volume of solvent II and applied to a cellulose column (60 × 4.3 cm, packed in solvent II). Elution was carried out with the same solvent and 12-mL fractions were collected. Fluorescent material appeared in tubes 65–74. Examination of individual fractions by paper chromatography in solvent III showed that no appreciable separation of the two components had been obtained. The fractions were combined and evaporated to a colorless glass (126 mg) which was chromatographed on four sheets of Whatman 3MM paper in solvent III.[4] The bands corresponding to the two components were cut out and eluted. The ammonium salts were converted to the free acids by cation exchange. Evaporation yielded 86.8 mg of the material of R_f 0.71 and 9.4 mg of the lower running contaminant as colorless glasses. The former, **3i**, was crystallized by addition of Me_2CO to its solution in the minimum volume of *i*-PrOH: yield 47%; needles from Me_2CO–*i*-PrOH; mp dec from 140 °C.

2-Chloro-N^6-methyladenosine 5′-Phosphate (3j). (Scale of reaction: 3 mmol.) The cation-exchange chromatographic step was omitted. The product was isolated as the amorphous free acid from the anion-exchange column. Dropwise treatment of a concentrated aqueous solution with ethanol resulted in white rosettes: mp dec from 160 °C (75%).

2-Methylthio-N^6-methyladenosine 5′-Phosphate (3k). (Scale of reaction: 1 mmol.) Upon acidification of the filtrate from the alkaline hydrolysis a large proportion of the isopropylidene nucleoside 5′-phosphate separated out as a white solid which failed to redissolve on heating at 70–80 °C for 2 h. It was removed by filtration, dissolved in 80% HCOOH, and allowed to stand for 8 h. Meanwhile the filtrate was neutralized with LiOH and worked up to remove precipitated Li_3PO_4. To it was added a neutralized (LiOH) solution of the residue remaining after evaporation of the 8-h hydrolysis mixture and the combination was purified by ion-exchange chromatography in the usual way to yield 75% free acid: mp 190–197 °C dec; recrystallized from H_2O; needles; mp 192–197 °C dec.

2-Chloro- and 2-Methylthioadenosine 5′-Phosphorothioates (4b,e). Nucleoside **1b** or **1e** (1 mmol) was dissolved in triethyl phosphate (2.5 mL) by rapidly heating the solvent almost to its boiling point. The mixture was cooled to 25 °C, $PSCl_3$ (0.3 mL, 3 mmol) was added, and the solution was kept at 3 °C under anhydrous conditions for 12 h. It was then treated with additional $PSCl_3$ (3 mmol) and allowed to stand for a further 12 h at 3 °C. Barium acetate solution (20%, 10 mL) was added and, after 1 h at 25 °C, the pH of the solution was adjusted to ca. 9 with Et_3N. EtOH (30 mL) was added and the resulting white precipitate was collected by centrifugation and washed with 70% aqueous EtOH until no further UV-absorbing material could be extracted. The pellet was then exhaustively washed with H_2O (ca. 200 mL) until all the nucleotide material had been removed. This aqueous extract was passed through a column of DEAE cellulose (HCO_3^-, 2.5 × 20 cm) and followed by 250 mL of H_2O. Elution with a linear gradient (0–0.4 M in 1 L) of ammonium bicarbonate produced a single chromatographically homogeneous peak which emerged

in 15-mL fractions 32–55 (**4b**) or 40–57 (**4e**). The 5′-phosphorothioates were both isolated as white freeze-dried solids in 42% yield.

The compounds were further purified by preparative paper chromatography in solvent III[4] and recovered (>95%) after lyophilization as the monoammonium salts.

2-Ethylthioadenosine 5′-phosphorothioate (4f) was prepared from **1f** in a manner similar to the above and was isolated after ion-exchange and paper chromatography in 26% yield.

Synthesis of 5′-*O*-Sulfamoyl Derivatives 5a,b,e. 2′,3′-*O*-Isopropylidene nucleoside (**2a**,**b**[6] or **2e**) was dissolved in 1,2-dimethoxyethane (50–70 mL) and treated with NaH. After 0.5–1 h of stirring, sulfamoyl chloride in 1,2-dimethoxyethane (20 mL) was added dropwise over 20 min and the suspension was stirred for a further 4 h at 25 ° C. The relative quantities of reactants used are given in Table III. Absolute EtOH (10 mL) was added and after stirring for 10 min the mixture was evaporated to dryness. The residue was suspended in water, neutralized (0.1 M HCl), and extracted first with *n*-hexane and then with EtOAc (in the case of the methylthio derivative the residue was extracted with hexane before being dissolved in 50% EtOH and neutralized with HCl; this solution was evaporated to dryness and the dried solid residue was extracted with EtOAc). Solvent was removed from the EtOAc extract in vacuo and the residue was treated with 20% (50% for **5e**) HCOOH for 48 h. Volatiles were removed and the product was crystallized from water.

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Studies on Drug Metabolism by Use of Isotopes. 23.[1] Metabolic Study of 1-Butyryl-4-cinnamylpiperazine in the Rat during Development of Tolerance by Using Two Kinds of Deuterium-Labeled Forms

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Mass fragmentographic determination of the plasma, brain, and liver levels of 1-butyryl-4-cinnamylpiperazine (I) and its metabolites in tolerant and nontolerant rats was carried out by using two kinds of deuterium-labeled compounds. 1-Butyryl-4-[α-d_2]cinnamylpiperazine (I-d_2) was used to develop tolerance and 1-butyryl-4-[arom-d_5]cinnamylpiperazine (I-d_5) to clarify the metabolic fate of I after I-d_2 administration. The present method allowed the clarification of the metabolism of I-d_2 and I-d_5 independently. In tolerant rats, the brain and liver levels were lower for I-d_5 and higher for I-d_5 metabolites than in nontolerant rats. There was no significant difference in the plasma levels of I-d_5 between tolerant and nontolerant rats. Our data seem to suggest that development of tolerance to I results from a more rapid metabolism of the drug due to hepatic enzyme induction.

Several methods have been reported to investigate the effects of repeated administration of a drug on its own metabolism. For example, by spectrophotometry[2,3] the plasma half-lives of drug repeatedly and singly administered to experimental animals have been compared. The plasma, brain, and tissue levels[4-8] and the urinary excretion[9] of drugs and their metabolites in singly and repeatedly administered animals have been determined by a radioactive isotope tracer technique. By these methods, however, simultaneous determination of the metabolic fate of a drug repeatedly administered and that of the same drug finally administered cannot be performed.

The use of stable isotopes together with mass spectrometry has provided a specific and sensitive method for the simultaneous determination of labeled and unlabeled drug in plasma.[10] This method is of great value in the study of steady-state pharmacokinetics of drugs.[11]

In this paper we propose the following experimental method of using a stable isotope tracer technique in which the metabolism of drug repeatedly administered and the metabolism of the same drug finally administered can be clarified simultaneously. The main idea is to use two forms of stable isotope-labeled drug, e.g., drug A-d_2 and drug A-d_5. After repeated administration of drug A-d_2, a single dose of drug A-d_5 is given. Quantitative determination of drug A-d_2 and drug A-d_5, and their respective metabolites, is then performed simultaneously by a gas chromatograph–mass spectrometer–multiple ion detector (GC–MS–MID). As internal standard for the GC–MS–MID measurements, unlabeled compounds of the drug and its

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